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Induction of DNA Breakage in U937 Cells by Oxazaphosphorines*

Lidia MAZUR, Małgorzata OPYDO-CHANEK, Marta STOJAK, Jarosław BARAN, and Ulf NIEMEYER

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Oxazaphosphorines are a class of DNA alkylating agents. The aim of the present study was to compare the possible influence of three new generation oxazaphosphorines, D-17272 (mafosfamide cyclohexylamine salt), D-18864 (4-hydro-peroxy-cyclophosphamide), and D-19575 (glufosfamide, β-D-glucose-isophosphoramide mustard) on DNA damage induction in the human histiocytic lymphoma U937 cells. The flow cytometry APO-BRDU^{TM} assay, based on the TUNEL method, was used for the *in situ* detection of DNA strand breaks. After exposure of U937 cells to the oxazaphosphorines, the patterns of temporary changes in the frequency of TUNEL positive U937 cells, expressing DNA breakage, were determined. The effects of the oxazaphosphorines on U937 cells were dependent on the agent tested and its dose, and the time intervals after the drug application. The different potential of D-17272, D-18864 and D-19575 to induce DNA strand breakage in the human histiocytic lymphoma U937 cells was shown.

Key words: Oxazaphosphorines, U937 cells, DNA strand breaks, TUNEL technique.

Lidia MAZUR, Małgorzata OPYDO-CHANEK, Marta STOJAK, Department of Experimental Hematology, Jagiellonian University, R. Ingardena 6, 30-060 Kraków, Poland.
E-mail: lidia.mazur@uj.edu.pl
malgorzata.opydo-chanek@uj.edu.pl
marta.stojak@uj.edu.pl
Jarosław BARAN, Department of Clinical Immunology, Polish-American Institute of Pediatrics, Jagiellonian University Medical College, Wielicka 265, 30-663 Kraków, Poland.
E-mail: mibaran@cyf-kr.edu.pl
Ulf NIEMEYER, NIOMECH part of IIT GmbH, University of Bielefeld, Universitätsstr. 25, 33615 Bielefeld, Germany.
E-mail: ulf_niemeyer@t-online.de

Oxazaphosphorines belong to a class of anticancer alkylating agents. The oxazaphosphorines are very useful and effective in the treatment of a wide variety of hematological and non-hematological malignancies. These therapeutic drugs include the commonly used cyclophosphamide and ifosfamide (BROCK 1989; ADIS INTERNATIONAL LIMITED 2005; ZHANG *et al.* 2005a, 2005b). Recently, additional oxazaphosphorine derivatives, such as mafosfamide cyclohexylamine salt, 4-hydroperoxy-cyclophosphamide and glufosfamide, have been synthesized and tested for the improved activity against different types of cancer cells (ENGEL *et al.* 2000; SEKER *et al.* 2000; ZHANG *et al.* 2005a; MISIURA 2006; LIANG *et al.* 2007). These novel oxazaphosphorines, and especially glufosfamide, have attracted much attention in a broad spectrum of cancer therapy. The modes of their action are still under experimental and clinical investigations (BRIASOULIS *et al.* 2000, 2003; BRÖKER & GIACCONE 2002; STYCZYŃSKI *et al.* 2002a, 2002b; VAN DEN BENT *et al.* 2003; DOLLNER *et al.* 2004; GIACCONE *et al.* 2004; ZHANG *et al.* 2005a, 2005b; AMMONS *et al.* 2007; CHIOREAN *et al*. 2008; GOLDSTEIN *et al.* 2008; MAZUR *et al.* 2008a, 2008b; CIULEANU *et al.* 2009).

There is a general consensus that DNA is the main target for a large number of anticancer drugs. The oxazaphosphorines are accepted to be DNA damaging agents. A better understanding of the action of these alkylating agents on DNA molecules of cancer cells is important for their optional use in chemotherapy (SEKER *et al.* 2000; BECKER *et al.* 2002).

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The aim of the present study was to compare the potential of three new generation oxazaphosphorines to induce DNA breakage in the human histiocytic lymphoma U937 cells. The *in situ* induction of DNA strand breaks in U937 cells subjected to the exposure to mafosfamide cyclohexylamine salt, 4-hydro-peroxy-cyclophosphamide and glufosfamide, was analyzed using the TUNEL method.

Material and Methods

Cells

Human histiocytic lymphoma U937 cells (American Type Culture Collection, Rockville, MD, USA) were maintained in RPMI 1640 (Gibco BRL Life Technologies) supplemented with 10% fetal calf serum (GIBCO BRL Life Technologies), 2 mM L-glutamine (Sigma Aldrich), and antibiotic antimycotic solution (Sigma Aldrich) containing 20 units of penicillin, 20 μg streptomycin and 0.05 μg amphotericin B. U937 cells were passaged every third day, by diluting to a concentration of $5x10^5$ cells/ml medium, and the cells grew exponentially at 37°C in an atmosphere of 5% CO_2 in air (HERAcell incubator, KendroLab). The cultures were periodically tested for *Mycoplasma* infection.

Chemicals

Mafosfamide cyclohexylamine salt (D-17272, CAS No. 84210-80-0), 4-hydro-peroxy-cyclophosphamide (D-18864, CAS No. 39800-16-3) and glufosfamide (D-19575, β-D-glucose-isophosphoramide mustard, CAS No. 132682-98-5) were obtained from NIOMECH (Bielefeld, Germany). D-17272, D-18864 and D-19575 were dissolved in aqua pro injectione (Polpharma, Poland). All solutions were freshly prepared directly before treatment of U937 cells.

Drug doses and cell treatment

After a dilution of the cell suspension to a density of $15x10^4$ cells/ml, U937 cells were subjected to 30 min exposure to the oxazaphosphorine drug. D-18864 was given at a dose of 5 μg/ml medium, D-19575 at a dose of 50 μg/ml medium, and D-17272 was applied at doses 5 μg/ml and 50 g/ml medium. The control material consisted of untreated cells. After 30 min treatment with the oxazaphosphorine agent, U937 cells were centrifuged for 10 min at 1000 rpm and the supernatant was discarded. Then the cells were washed in 2 ml of PBS (BioMed) and pelletted by centrifugation for 7 min. The wash and centrifugation were repeated once more and the cells resuspended in 5 ml of the complete RPMI 1640 medium.

Preparation procedure and fixation of cells

Samples of the cell suspension were taken at 24h, 48h and 72h after drug application. After 10 min centrifugation at 1000 rpm, and discarding the supernatant, the cells were washed twice in 2 ml of PBS and pelletted by centrifugation for 7 min. U937 cells were flushed out with PBS and dispersed by gentle pipetting. Finally, the cells were suspended in 500 μl of PBS. After adding 5 ml of 1% (w/v) formaldehyde (Lach-Ner) in PBS, the cell suspension was placed on ice for 15 min. Then, U937 cells were centrifuged for 7 min at 1000 rpm and the supernatant was discarded. The cells were washed in 2 ml of PBS and pelletted by centrifugation. The wash and centrifugation were repeated once more. The cells were resuspended in 500 μl of PBS, and after adding 5 ml of ice-cold 70 % (v/v) ethanol, stored at -20°C until use.

APO-BRDU™ assay

The APO-BRDU™ Kit (Calbiochem), a two color staining method for labeling DNA breaks and total cellular DNA was used. The 3' OH termini in the strand breaks can be analyzed by attaching FITC (fluorescein isothiocyanate)-labeled 5-bromo-2'-deoxyuridine 5'-triphosphate nucleotides (Br-dUTP), in a reaction catalyzed by exogenous terminal deoxynucleotidyl transferase (TdT). This reaction is known as TUNEL from TdT-mediated dUTP nick-end labeling. The incorporation of Br-dUTP into DNA strand breaks, detected by FITC-conjugated anti-BrdU antibody, is combined with staining of DNA with propidium iodide (PI) (DARŻYNKIEWICZ *et al.* 2001). The kit consisted of washing, reaction, and rinsing buffers for processing individual steps in the assay, TdT, Br-dUTP, fluorescein labeled anti-BrdU antibody for labeling DNA breaks and PI/RNase A solution for counter staining the total DNA.

APO-BRDU™ protocol

The leukemic cell suspension was centrifuged for 10 min at 1000 rpm and the 70% ethanol was removed by aspiration. The cell pellet was resuspended with 1 ml of wash buffer, centrifuged for 7 min at 1000 rpm and the supernatant removed. The wash buffer treatment was repeated and the cell pellet resuspended in 50 μl of the DNA labeling solution containing 10 μl of TdT reaction buffer, 0.75 μl of TdT enzyme, 8 μl of Br-dUTP,

and 32.25 μl of distilled H_2O. The cells were incubated in the DNA labeling solution for 60 min at 37°C in a temperature controlled bath. At the end of incubation time, 1 ml of rinse buffer was added to each tube, the cells centrifuged for 7 min at 1000 rpm and the supernatant removed by aspiration. Cell rinsing with 1 ml of the rinse buffer was repeated, and then the cell pellet was resuspended in 100 μl of the antibody solution containing 5 μl of fluorescein labeled anti-BrdU antibody suspended in 95 μl of the rinse buffer. The cells were incubated with the fluoresceinated antibody solution in the dark for 30 min at room temperature. Then, 300 μl of the propidium iodide/RNase A solution was added to the tube containing 100 μl of the antibody solution, and the cells were incubated in the dark for 30 min at room temperature.

Flow cytometry analysis of leukemic cells

The fluorescence of individual U937 cells was measured by a flow cytometer (FACS Calibur, Beckton Dickinson). Green fluorescence of FITC-conjugated anti-BrdU antibody was measured at 530-nm and red fluorescence of propidium iodide at >600 nm. At least 5 000 cells were analyzed per one sample.

Results

The effects of three oxazaphosphorines, D-17272, D-18864, and D-19575, on DNA degradation in U937 cells, were compared. Using the flow cytometry APO-BRDU^{TM} assay, based on the *in situ* detection of DNA strand breaks, temporary changes in the frequency of TUNEL positive U937 cells were determined at 24h, 48h, and 72h after the application of the oxazaphosphorine agent (Table 1, Fig. 1).

The frequency of DNA strand breaks was found to be smaller in U937 cells treated with D-17272, at a dose of 5 μg/ml medium, than in the cells exposed to D-18864, at the same dose. The frequency

Table 1

Effects of the oxazaphosphorines on DNA strand breaks – induction in U937 cells

Time intervals after cell exposure to oxazaphosphorines			24h	48h	72h
Experimental groups			Frequency of U937 cells expressing DNA strand breaks		
No.	drug given	drug dose	Mean ± SD	Mean ± SD	Mean ± SD
I	D-18864	5 μg/ml	2, 3, 4, 5, 72h 38.27 ± 1.12	2, 3, 4, 5 42.67 ± 5.41	2, 3, 4, 5, 24h 47.59 ± 6.91
II	D-17272	5 μg/ml	1, 5, 48h, 72h 7.91 ± 1.91	1, 3, 5, 24h 5.41 ± 0.42	1, 3, 4, 5, 24h 5.01 ± 0.80
III	D-17272	50 μg/ml	1, 5, 48h, 72h 11.49 ± 1.95	1, 2, 4, 5, 24h, 72h 63.83 ± 4.61	1, 2, 4, 5, 24h, 48h 76.02 ± 2.71
IV	D-19575	50 μg/ml	1, 5 9.11 ± 2.56	1, 3, 5, 72h 6.76 ± 1.42	1, 2, 3, 5, 48h 11.79 ± 1.70
V	control		1, 2, 3, 4 2.23 ± 0.16	1, 2, 3, 4 2.48 ± 0.21	1, 2, 3, 4 2.29 ± 0.39

Statistically significant differences at $P<0.05$

Differences between groups: different from Group I – 1, Group II – 2; Group III – 3; Group IV – 4; Group V – 5.

Differences within each group: different from 24h – 24h; 48h – 48h; 72h – 72h.

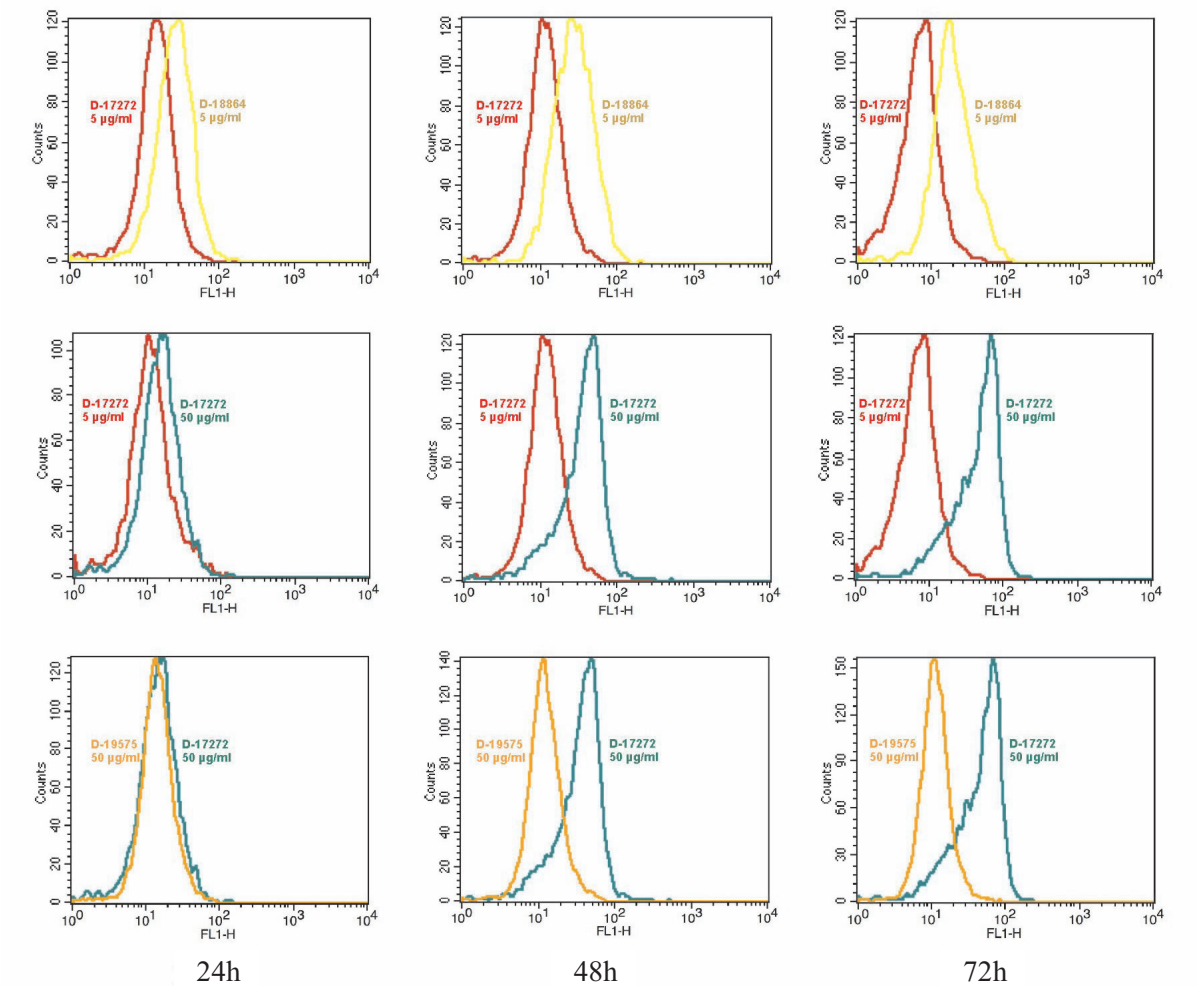

Fig. 1. *In situ* detection of DNA strand breaks in U937 cells after application of D-17272, D-18864 and D-19575, using flow cytometry APO-BRDU^{TM}assay. Histograms of FITC green fluorescence intensities of U937 cells treated with: D-18864 (5 μg/ml medium) – yellow line; D-17272 (5 μg/ml medium) – red line; D-17272 (50 μg/ml medium) – green line; D-19575 (50 μg/ml medium) – orange line. FL1-H – BrdUTP-FITC.

of TUNEL positive cells, assessed at 48h and 72h after the exposure of U937 cells to D-17272, appeared to be respectively greater when the oxazaphosphorine agent was applied at the higher dose of 50 μg/ml than at the lower one of 5 μg/ml medium. The frequency of DNA strand breaks observed at 48h and 72h after D-19575 application, at a dose of 50 μg/ml medium, was respectively smaller as compared with that found after the treatment of U937 cells with D-17272, at the same dose. In comparison with the controls, the frequency of U937 cells expressing DNA strand breaks increased in all remaining experimental groups at all time intervals after the oxazaphosphorine application.

Discussion

The results of the present study have shown the influence of three new generation oxazaphosphorines, D-17272, D-18864 and D-19575, on DNA breakage induction in the human histiocytic lymphoma U937 cells. The different patterns of temporary changes in the frequency of U937 cells expressing DNA strand breaks, were found at 24, 48 and 72h after the alkylating drug application. DNA degradation was dependent on the compound tested and its dose, and the time intervals after the exposure of U937 cells to the oxazaphosphorine agent.

The mechanisms of action of D-17272, D-18864 and D-19575, have not yet been completely explained (BODY & YULE 2000; ZHANG *et al.* 2005a). However, it is known that D-17272, mafosfamide cyclohexylamine salt, generates its active principle 4-hydroxy-cyclophosphamide (4-OH-CP) which degrades to phosphoramide mustard and acrolein. D-18864, that is 4-hydro-peroxy-cyclophosphamide, generates 4-OH-CP. Thus, phosphoramide mustard and acrolein are the major ultimate alkylating agents of the pro-drugs, D-17272 and D-18864

(BODY & YULE 2000; KEHRER & BISWAL 2000; ZHANG *et al.* 2005a, 2005b). Unlike these prodrugs, D-19575, β-D-glucose-isophosphoramide mustard, contains the directly active alkylating moiety, isophosphoramide mustard (IPM) linked to a β-D-glucose-molecule. Inside the cell, D-19575 hydrolyzes to IPM and β-D-beta-D-glucose (POHL *et al.* 1995; VEYHL *et al.* 1998; ENGEL *et al.* 2000; LIANG *et al.* 2007).

The cytotoxic effects of the oxazaphosphorines are thought to be dependent mainly on DNA alkylating properties. The active alkylating agents can bind to a variety of molecules, but the most important site of their binding is DNA. These active compounds can react with phosphate, amino, and hydroxyl groups of the bases of nucleic acids. The ultimate alkylating mustards from the oxazaphosphorines are the predominant metabolites that cause DNA damage such as adducts and crosslinks, and DNA strand breaks (SEKER *et al.* 2000; BECKER *et al.* 2002; ZHANG *et al.* 2005b). It is known that DNA damage can trigger programmed cell death. However, the mechanisms responsible for the activation of cellular death pathways leading to programmed cell death, due to the adduct and crosslink formation, and DNA breakage induction, have yet to be fully elucidated (GUIMARAES & LINDEN 2004; ZHANG *et al.* 2005a, 2005b; GOLDSTEIN *et al.* 2008).

The modes of action of D-17272, D-18864 and D-19575 are suggested to be closely related to the pharmacokinetic, pharmacodynamic, and cytotoxic properties of these three oxazaphosphorine agents. It is believed that the oxazaphosphorine metabolism and transport have a major impact on pharmacokinetic variability, the pharmacokinetic--pharmacodynamic relationship, and cytotoxicity (MOORE 1991; POHL *et al.* 1995; STUBEN *et al.* 1996; VEYHL *et al.* 1998; BODY & YULE 2000; ENGEL *et al.* 2000; BRIASOULIS *et al.* 2003; GIACCONE *et al.* 2004; ZHANG *et al.* 2005a, 2005b).

To summarize, the oxazaphosphorines D-17272, D-18864 and D-19575 distinctly affect DNA breakage formation in the human histiocytic lymphoma U937 cells. The results of the present investigation are the first data comparing the potential of these three alkylating agents, mafosfamide cyclohexylamine salt, 4-hydro-peroxy-cyclophosphamide, and glufosfamide, to induce DNA strand breaks in the malignant hematopoietic cells. An elucidation of the mechanisms and processes responsible for the different extent of DNA damage occurring in various types of cancer cells, following their exposure to the oxazaphosphorine agents, can facilitate the development of new therapeutic strategies.

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